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Note

Isolation of a thermophilic alkaline phosphatase by either hydrophobic or Procion red Sepharose chromatography

DANNY INY*, JOSEPH SOFER and ALEX PINSKY
Department of Life Sciences, Faculty of Natural Sciences, Bar-Ilan University, Ramat-Gan (Israel)
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Affinity chromatography has become the most efficient method for the purification of active proteins, both with respect to the yield and the extent of enrichment[1]. In general, an analogue of the substrate is bonded to an insoluble support such as Sepharose and inactive proteins are eluted under mild solutions, the active factor is eluted under more drastic conditions, usually a higher salt concentration.

When this method was applied to alkaline phosphatase, little success was achieved. Phenylalanine[2,3], phosphoric acid[3,4] and arsonic acid[5,6] gave poor results. The yield of reagent was poor, and the efficiency of the procedure was low.

Triazine dyes have been used successfully in the purification of many enzymes, especially kinases[7,8] and dehydrogenases[9]. Their action is similar to that of affinity chromatography. The nucleotide binding fold of the enzyme to be purified apparently reacts with the dye and relatively mild conditions such as NAD, NADH, ATP, phosphate or an increase in salt concentration suffices to elute the active principle. Thus calf intestinal alkaline phosphatase was eluted from a red Sepharose column with a phosphate gradient of 0–25 m*M*, yielding a final purification of 2500-fold[8]. An organophosphate hydrolysing phosphatase was purified from a Cibacron blue column by a change in the pH of the eluent[10]. In the case of the thermophilic alkaline phosphatase described here, elution by much more drastic means such as 4 *M* guanidine hydrochloride was necessary.

Agarose gels substituted with amino alkanes containing four or more carbon atoms retained phosphorylase[11,12]. Agarose substituted with diamines could be used in the purification of glycogen synthetase[12]. From these experiments, it appeared that a protein with hydrophobic pockets will attach itself to a hydrocarbon chain on an insoluble support[13]. Elution is generally achieved by a change in a parameter such as pH or salt concentration[13]. Thus Nitzan and Michalsky[14] succeeded in purifying diphtheria toxin by hydrophobic chromatography. In the case of the thermophilic alkaline phosphatase described here, none of the above elution methods was successful and as described for the triazine dyes, only 4 *M* guanidine hydrochloride eluted the enzyme. This may be due to the thermophilic nature of the enzyme. Both purification procedures are now described.

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EXPERIMENTAL

The source of the alkaline phosphatase was a culture of a facultative thermophilic actinomycete grown at 46°C (found to be optimal) for 68–72 h on a rotary shaker. After centrifuging at 10 000 *g* for 30 min, the cells were suspended in 10 m*M* Tris buffer pH 7.5 containing 0.1 m*M* cobalt(II) sulphate, 2 m*M* magnesium chloride and 2 m*M* calcium acetate (starting buffer). The suspension was then sonicated four times, each time for 15 s. 2% Tween 20 was added and the product was homogenized in a Polytron homogenizer (Kinematica, Lucerne, Switzerland) and then slowly stirred with a magnetic stirrer for 1 h. This suspension was then centrifuged at 82 000 *g* (Spinco centrifuge) for 30 min and the supernatant was filtered through an Amicon filter PM30 (cut-off value 30 000). The retentate was used in the subsequent purifications.

Protein analysis

The protein content was determined by the Coomassie blue method[15] as modified by Gogstad and Krutens[16] or by the absorption at 280 nm.

Activity determination

The enzyme activity was determined on a *p*-nitrophenyl phosphate substrate as described by Schlesinger and Barret[17].

Electrophoresis

The electrophoretic patterns of the purified products were determined on 10% polyacrylamide gel[18] using the apparatus of Avtalion[19,20]. The pH was 8.9 and the voltage and amperage were intially 100 V and 50 mA, respectively, raised to 200 V and 100 mA after some minutes. The duration of the experiment was 4 h.

Purification

DEAE-cellulose column. The crude enzyme was applied to a DEAE-cellulose column (8 cm × 2 cm) which had been equilibrated with the starting buffer until the eluent showed no absorption at 280 nm. Elution was with a stepwise gradient of 45 ml each of 0.2, 0.4 and 0.8 *M* potassium chloride dissolved in the starting buffer.

Octyl-Sepharose column. The active fraction from the DEAE-cellulose column was applied to and Octyl Sepharose column (10 cm × 2 cm) which had been rinsed with the starting buffer until the elute showed no absorption at 280 nm. The column was stoppered for 5 min and then rinsed with the starting buffer to remove inactive protein. It was then eluted with 4 *M* guanidine hydrochloride dissolved in the starting buffer.

Pentyl-Sepharose column. A 30-g amount of Sepharose 4B was rinsed with cold water to remove preservatives. It was then rinsed with 100 ml of 2 *M* potassium carbonate and then placed in a beaker containing crushed ice. The Sepharose was mixed with 30 ml 2 *M* potassium carbonate to form a suspension. Cyanogen bromide in acetonitrile (1 g/ml) was added (1.5 ml for each 10 ml suspension). After 3 min of vigorous stirring, the Sepharose was rinsed with cold distilled water and then with 50 ml cold borate buffer (0.05 *M*, pH 9) and dried. A 2-ml volume of pentylamine was added to 40 ml of 0.05 *M* borate buffer pH 9.0. The pH was readjusted to 9.0

with 6 *M* hydrochloric acid. The activated Sepharose (30 g) was added to this solution with constant stirring. After 5 h of stirring at room temperature, the Sepharose was rinsed to remove the excess of pentylamine. This pentylamine Sepharose can be stored in 0.1 *M* acetic acid.

The Pentyl-Sepharose was rinsed with starting buffer, the enzyme was applied and the column was then stoppered for about 10 min. Inactive protein was eluted with the starting buffer which was added until the eluate had no absorption at 280 nm. The column was then eluted with 45 ml of 0.5 *M* potassium chloride in the starting buffer.

Red Sepharose chromatography

The active fraction from the Pentyl Sepharose column was applied to a red Sepharose column (10 cm × 2.5 cm). The column was stoppered for about 5 min and rinsed with the starting buffer as above. It was eluted with 40 ml of 4 *M* guanidine hydrochloride in the starting buffer.

RESULTS

DEAE-cellulose and Octyl-Sepharose chromatography

The active enzyme was eluted from the DEAE-cellulose column with 0.8 *M* potassium chloride in the starting buffer. Lower concentrations of potassium chloride removed inactive protein. When the active fraction from this column was applied to an Octyl-Sepharose column, drastic means were need to elute the enzyme. The eluent was 4 *M* guanidine hydrochloride in the starting buffer. High salt or lower guanidine concentrations were ineffective.

Table I gives a typically recovery experiment. An enrichment of 177-fold and a recovery of 27% were achieved.

TABLE I

PURIFICATION OF THERMOPHILIC ALKALINE PHOSPHATASE BY HYDROPHOBIC CHROMATOGRAPHY

The filtration was through an Amicon PM30 membrane (cut-off value 30 000). The eluent in DEAE-cellulose chromatography was a stepwise gradient from 0.2 to 0.8 *M* potassium chloride. The hydrophobic chromatography was on Octyl-Sepharose and the eluent was 4 *M* guanidine hydrochloride.

Step	*Volume (ml)*	*Total protein (mg)*	*Total activity (u/min)*	*Specific activity (u/mg protein)*	*Enrichment*	*Yield (%)*
Membrane filtration	100	450	11 800	26	1	100
DEAE-cellulose chromatography	176	8.8	4752	540	21	40
Hydrophobic chromatography	141	0.7	3243	4600	177	27

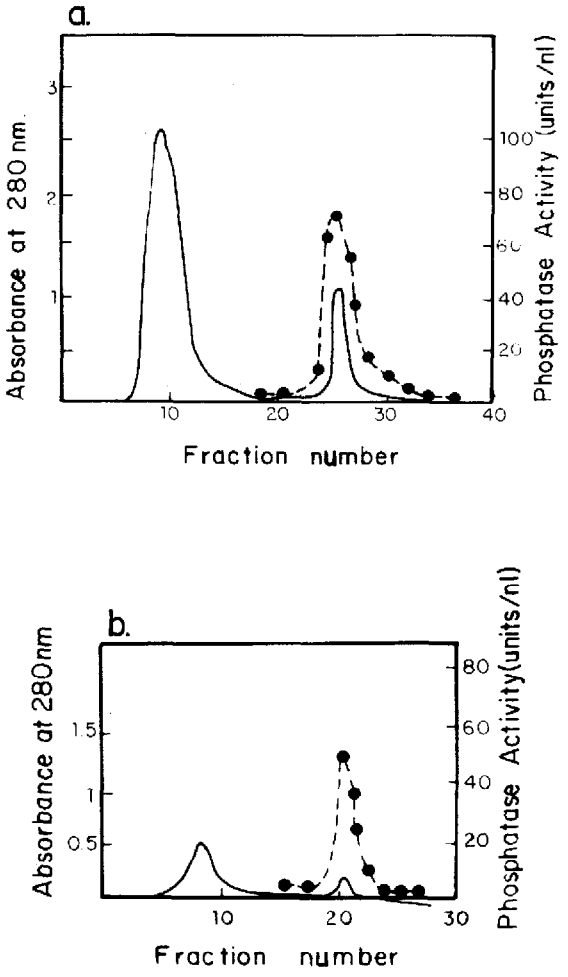

Fig. 1. Elution patterns of a thermophilic alkaline phosphatase: (a) on a Pentyl-Sepharose column after elution with 0.5 *M* potassium chloride in 10 m*M* Tris buffer pH 7.5 containing 0.1 m*M* cobalt(II) sulphate, 2 m*M* magnesium chloride and 2 m*M* calcium acetate; (b) on a red Sepharose column after elution with 4 *M* guanidine hydrochloride in the above buffer.

Pentyl- and red Sepharose chromatography

The active enzyme was eluted from a Pentyl Sepharose column (10 cm × 2 cm) with 0.5 *M* potassium chloride in the starting buffer. In the second chromatographic stage on a red Sepharose column (10 cm × 2.5 cm) the active fraction was eluted with 4 *M* guanidine hydrochloride in the starting buffer only. Lower guanidine salt concentrations did not release the enzyme. Fig. 1 shows a typical purification experiment. An enrichment of 102-fold and a yield of 37% were obtained. Table II gives a typical recovery experiment.

Fig. 2 shows the electrophoretic patterns of both purified fractions. The product from hydrophobic chromatography was the purest.

TABLE II

PURIFICATION OF THERMOPHILIC ALKALINE PHOSPHATASE BY PROCION RED CHROMATOGRAPHY

The filtration was through an Amicon PM30 membrane (cut-off value 30 000). The eluent in Pentyl-Sepharose chromatography was 0.5 *M* potassium chloride. The eluent for the red Sepharose chromatography was 4 *M* guanidine hydrochloride.

Step	*Volume (ml)*	*Total protein (mg)*	*Total activity (u/min)*	*Specific activity (u/mg protein)*	*Enrichment*	*Yield (%)*
Membrane filtration	4	16	644	40	1	100
Pentyl-Sepharose chromatography	9	0.99	450	455	11	69
Red Sepharose chromatography	6	0.06	240	4100	102	37

Fig. 2. Electrophoretic patterns of the thermophilic actinomycete alkaline phosphatase on 10% polyacrylamide in Tris–glycine buffer pH 8.9 at a final voltage of 200 V and current of 100 mA. Duration of the analysis: 4 h. Fractions: A, purified on DEAE-cellulose and Octyl-cellulose columns; B, purified on Pentyl-Sepharose and red Sepharose columns.

DISCUSSION

In the experiments described, hydrophobic chromatography on Pentyl-Sepharose as compared with Octyl-Sepharose showed that the binding was stronger

with the larger alkane. Whereas 0.5 *M* potassium chloride could elute the enzyme from the Pentyl Sepharose, nothing less that 4 *M* guanidine hydrochloride was successful in removing the enzyme from the Octyl-Sepharose. The hydrophobic interactions occurred between the available hydrophobic regions in the enzyme and the hydrocarbon chain of the Sepharose. Shaltiel emphasized this point, showing that the binding becomes stronger as the length of the chain increases[12] and that a column where only ammonia was attached to the Sepharose did not bind any of his enzyme[11].

The second scheme using red Sepharose is often called affinity chromatography. The dye is a triazine compound and serves as an analogue for nucleotides so that the protein will bind to it at its nucleotide binding site. This method is preferable to other chromatographic methods since the enrichment is obtained rapidly in one step instead of the lengthy procedures in other chromatographic techniques. The column is stable for long periods and can be reused many times; one active column is over 3 years old. A comparison of thermophilic alkaline phosphatase studied in this work with the mesophilic animal enzyme shows that the latter could be eluted from a Procion red Sepharose column with phosphate[10] but, as stated above, the thermophilic enzyme was not eluted and only drastic means, 4 *M* guanidine hydrochloride which completely denatured the mesophilic enzyme, was successful in eluting the active enzyme, which remained active for some time (4 h) in the presence of this reagent.

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